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Template Assembled Synthetic Proteins: Condensation of a Multifunctional Peptide to a Topological Template via Chemoselective Ligation

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Keywords: Template Assembled Synthetic Proteins, chemoselective ligation, multifunctional peptide, topological template, oxime bond formation.

Abbreviations: TASP Template Assembled Synthetic Proteins; SPPS Solid Phase Peptide Synthesis; Boc tert. butyl oxycarbonyl; Pmc 2,2,5,7,8-penta methyl chroman-6-sulphonyl; TFA trifluoroacetic acid; r.t. room temperature; RP-HPLC reverse phase high pressure liquid chromatography; LDI-MS laser desorption ionisation mass spectrometry.

Abstract: A chemoselective ligation via oxime bond formation is used for the chemical synthesis of template assembled peptides according to the TASP (Template Assembled Synthetic Proteins) approach. Aminooxyacetylation of the multifunctional partial sequence Lys- Arg- Asp- Ser of lactoferrin and subsequent condensation in aqueous solution with a topological template containing four selectively addressable aldehyde functions as attachment sites gives readily access to the TASP molecule.

De novo design of proteins has become an attractive goal in synthetic peptide chemistry[1]. In particular, the covalent binding of secondary structure forming, amphiphilic oligopeptides to topological templates according to the TASP approach represents an elegant tool for overcoming the protein folding problem, one of the major obstacles encountered in the design of sequences thought to fold in a predetermined three-dimensional structure. A number of 4α-helix bundle TASP molecules has been prepared either by stepwise solid phase peptide synthesis (SPPS)[2] or by convergent strategies[3]. Both synthetic approaches suffer from some fundamental limitations, i.e. the elaborous purification protocols of the target molecule[2] or the low solubility of fully protected peptide fragments[3]. Recently, chemoselective ligation methods have been proposed for the attachment of unprotected peptides to carrier molecules[4]. The application of these techniques in the preparation of TASP molecules can, in principle, overcome these limitations and give access to the efficient and reliable synthesis of these protein- like molecules. As a prototype, we describe here the chemoselective ligation of a multifunctional peptide block to a topological template[5] via selective oxime bond formation.

In our ongoing studies on biological and pharmacological properties of template assembled bioactive peptides, the partial sequence Lys-Arg-Asp-Ser (KRDS) of the milk protein lactoferrin[6] exhibiting potential antithrombotic activity was covalently linked to a topological template carrying four attachment sites. Due to the presence of a multitude of functional groups in the amino acid side chains (i.e. guanidino, amino, hydroxy and carboxylic groups) this peptide appeared to be an ideal prototype for studying the chemoselectivity of the ligation mode.

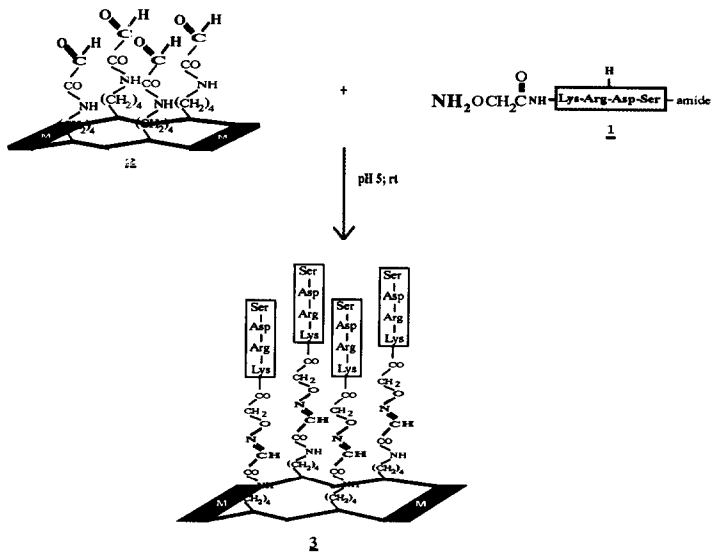

Figure 1: *Chemoselective ligation of a completely deprotected peptide to a topological template via oxime bond formation (see text).*

For the condensation of the unprotected peptide fragments to the cyclic carrier molecule (template) via oxime bond formation, the two building blocks **1** and **2** were assembled by SPPS and functionalized by an aminooxyacetyl group and an aldehyde group as depicted in Figure 1.

To this end, Boc-aminooxyacetic acid[7] was coupled to the N-terminal amino acid of the polymer bound peptide chain

H-Lys(Boc)-Arg(Pmc)-Asp(O^{t}Bu)-Ser(^{t}Bu)- resin.

After cleavage from the resin (50% TFA, 30 min, r.t.), the completely deprotected aminooxyacetylated peptide **1** was purified by RP- HPLC on a Vydac C_{18} column to homogeneity. In parallel, the attachment sites (ε-amino group of lysine) on the template molecule[5a]

c(Lys-Gly-Lys-**M**-Lys-Gly-Lys-**M**)

M= amino methyl naphthoic acid as dipeptide mimetic[5a]] are transformed to aldehyde functions by reaction with glyoxylic acid 1,1 diethylacetal and subsequent hydrolysis[8] to yield compound **2**. The condensation step of the water soluble peptide block to the template molecule was achieved under mild conditions in sodium acetate buffer at pH 5 using **1** in a threefold excess with respect to the functional groups on the template[9]. Due to the kinetically stable oxime bond[10], selective attachment of the peptide via its N-terminus to the template proceeded quantitatively as detected by analytical RP-HPLC (Figure 2). The target

molecule **3** was separated from excess tetrapeptide by semi- preparative RP-HPLC and its chemical integrity established by LDI-MS ($M_{calc.}$: 3458) and amino acid analysis.

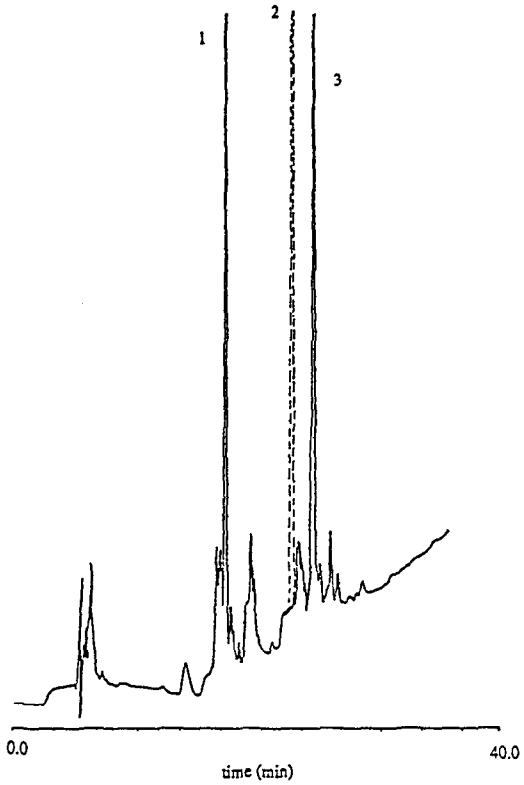

Figure 2: *Analytical HPLC of the reaction of H_2NOCH_2CO-KRDS (1) used in excess with the tetrakis aldehyde template (2), in aqueous solution at pH 5 after 15h. The product TASP is indicated (3). The dashed-line peak indicates the position of the template molecule.*

Preliminary assays indicate increased stability of the template attached peptides to enzymatic degradation and prolonged action in vivo. More detailed studies on the effect of the template upon the binding properties of KRDS to fibrinogen receptors are in progress.

In summary, the present methodology represents an interesting alternative for the rapid and reliable chemical synthesis of TASP molecules. Most notably, the combination of chemoselective ligation methods[5] with orthogonal protection techniques of template molecules allows for the efficient synthesis of TASP molecules featuring a variety of packing topologies and functional properties.

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7. Boc aminooxy acetic acid was prepared by reacting commercially available O-carboxymethyl-hydroxylamine hemihydrochloride (1 eq.) with 2 eq. of di-tert-butyl dicarbonate in MeOH/H_2O (3:2).

8. The hydrolysis of the tetrakis diethyl diacetal was performed by treatment of 1 mg template with 200µl 1N HCl, 100µl acetic acid and 200µl H_2O for 2h. After evaporation under reduced pressure the residue was redissolved in 200µl 1N HCl and 200µl H_2O and stirred for another 1h. Complete hydrolysis as followed by RP-HPLC was achieved by a third treatment of the template using 1N HCl and H_2O (200 µl each, 3h).

9. The condensation reaction proceeded as follows: the tetrakis aldehyde (**2**) was dissolved in 300µl 1M sodium acetate and the pH adjusted to 5 with acetic acid. A 3-fold excess of the tetrapeptide (**1**) (with respect to the aldehyde groups) in 1M sodium acetate (pH5) was added and stirred for 15h at room temperature.

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